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1 **Title**

2 **Enterobacteriaceae populations during experimental *Salmonella* infection in pigs**

3

4 **Running title**

5 **Enterobacteriaceae in *Salmonella*-infected pigs**

6

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25

25 **Abstract**

26 *Salmonella* infection might affect other intestinal Enterobacteriaceae populations and possible
27 correlations between single Enterobacteriaceae populations would help to predict subclinical
28 *Salmonella* infections in pigs. In one experimental setup, weaned piglets (n=40) were infected
29 with *Salmonella* and sacrificed at 3 h, 24 h, 72 h or 28 days post infection (p.i.). Dilutions of
30 intestinal contents and mucosal tissues were plated on agar plates for determinations of
31 Enterobacteriaceae. In another experimental setup, weaned piglets (n=12) were infected with
32 *Salmonella* and probed over a period of 28 days p.i., and dilutions of rectal contents were also
33 tested for Enterobacteriaceae populations. The occurrence of single Enterobacteriaceae
34 populations was correlated with the occurrence of other tested Enterobacteriaceae populations
35 as well as with clinical parameters of the piglets.

36 *Salmonella* (infection strain), *E. coli* (hemolytic and non-hemolytic) and another six non-*E.*
37 *coli*/non-*Salmonella* Enterobacteriaceae (NENSE) genera with eight species were identified.
38 In general, the absolute numbers of *E. coli*, *Salmonella* and NENSE populations decreased
39 with increasing age of the animals. In the jejunum, the numbers of NENSE, *E. coli* and
40 *Salmonella* were all highly positively correlated with each other. The occurrence of hemolytic
41 *E. coli* had no obvious effects on the occurrence of other Enterobacteriaceae. Furthermore,
42 only few associations of Enterobacteriaceae populations with clinical parameters were
43 observed.

44 In conclusion, we did not observe evidences for either a competition between or benefits for
45 specific Enterobacteriaceae populations during *Salmonella* infection indicating that changes
46 in the composition of the intestinal Enterobacteriaceae microflora are not useful indicators of
47 subclinical *Salmonella* infections.

48

48 **Key words**49 pig, *Salmonella*, *E. coli*, Enterobacteriaceae, intestine, infection

50

51 **Abbreviations**52 NENSE non-*E. coli*/non-*Salmonella* Enterobacteriaceae53 NSE non-*Salmonella* Enterobacteriaceae

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54 **Introduction**

55 *Salmonella* is one of the most important causes of human gastroenteritis worldwide. In
56 Germany, a total of 42,851 human Salmonellosis cases were reported in 2008
57 (<http://www3.rki.de/SurvStat/>; Robert Koch Institute, Berlin, Germany). The consumption of
58 contaminated pork and pork products are one of the main sources for *Salmonella* infections
59 (Steinbach and Hartung, 1999). Pork and pork products are commonly contaminated with
60 *Salmonella* during processing of meat from *Salmonella*-infected animals. *Salmonella*
61 infections have been reported to be widespread in pig farms (Berends et al., 1996), and
62 infected pigs can carry *Salmonella* without being recognized as carriers harboring the high
63 risk for transmission of *Salmonella* via the food production chain to humans. Thus, from the
64 vantage point of human nutrition most critical *Salmonella* infections in pigs are
65 asymptomatic, subclinical infections.

66 After infection of the host, *Salmonella* pathogenesis is characterized by three phases:
67 the initial colonization of the intestine, the invasion of enterocytes, and finally the
68 dissemination to lymph nodes and other organs (Darwin and Miller, 1999). During the initial
69 colonization phase, *Salmonella* must compete with the autochthonous microflora for nutrients
70 and attachment/adhesion sites (Baba et al., 1991; Snoeyenbos et al., 1978). During the second
71 phase of *Salmonella* infection, invasion mechanisms and intraepithelial growth might also be
72 affected by other, resident bacteria including other Enterobacteriaceae (Hudault et al., 2001;
73 Kleta et al., 2006; Tsai et al., 2005). It might therefore be expected that there might be
74 correlations between the occurrence and/or persistence of *Salmonella* in intestinal contents
75 and the mucosa through the presence of other Enterobacteriaceae species.

76 In this study we characterized populations of Enterobacteriaceae in the porcine intestine. The
77 main focus of the study was to examine interactions between *Salmonella* and other non-
78 *Salmonella* Enterobacteriaceae (NSE) during experimental, subclinical *Salmonella* infections.

79

79 **Animals, materials and methods**

80 **Animal infection study.** The animal study was approved by the local animal welfare
81 committee of the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit
82 (LAGetSi), Berlin, Germany (No. G0037/02). Litters were infected subsequently within a one
83 year period. Piglets from five litters (hybrids of Deutsche Landrasse and Duroc) were housed
84 in groups after weaning (28 days post partum (p.p.). Piglets had *ad libitum* access to liquid
85 feed with the essential components wheat and soybean. No antibiotics were administered to
86 piglets and their sows for at least three months prior to the trial (Szabo et al., 2009).

87 On day 29 p.p., piglets were sedated by intramuscular application of 1.0 mg/kg
88 azaperon and challenged with *Salmonella* Typhimurium DT104 intragastrically using a
89 stomach tube. The *Salmonella* Typhimurium DT104 (BB440) strain used for the infection
90 study was originally isolated from a pig with sepsis. A nalidixic acid-resistant derivative
91 transformed with a GFP-expressing plasmid, pFVP25.1, was used for the infections. The
92 nalidixin acid-resistance simplified the reisolation of *Salmonella* from fecal and tissue
93 samples. The GFP-expressing plasmid was introduced into the *Salmonella* strain for tracing
94 *Salmonella* infection routes in organs (lymph nodes, spleen etc.) of animals by fluorescence
95 analysis in other studies as part of the Deutsche Forschungsgemeinschaft (DFG) Research
96 Unit “An Integrative Analysis of Mechanisms of Probiotic Action in Pigs” network. The
97 GFP-expressing plasmid had no impact on the virulence of the strain which was tested in
98 preliminary studies (data not shown). Extensive data from network partners are not included
99 in this manuscript as they are the subject of other publications.

100 For infection, *Salmonella* was cultured in Luria-Broth (LB) at 37°C overnight. The
101 following day, 1 ml was inoculated into 100 ml pre-warmed LB and grown at 37°C to an
102 optical density (OD) of 2.0, corresponding to an average final count of approximately 3×10^9
103 /ml. Each pig was infected with 2 ml of this culture mixed with 8 ml Buffered Peptone

104 Water (BPW, Merck, Darmstadt, Germany). The infection dose of approximately 6×10^9
105 bacteria/ml was chosen to cause only subclinical or mild Salmonellosis.

106 The study consisted of two parts. In the first experimental part (1), from each of five
107 litters, a total of eight weaned piglets were infected with the *Salmonella* strain. Two infected
108 piglets from each litter were randomly selected and sacrificed at 3h, 24h, 72h and 28 days
109 post infection (p.i.). These 40 sacrificed animals were tested for Enterobacteriaceae
110 populations of the two intestinal compartments “content” and “mucosa” from the two
111 intestinal sections “jejunum” and the “colon”. Mucosa-associated bacteria included adherent
112 as well as intracellular bacteria. Evaluation of the consistency of intestinal contents and
113 additionally of feces (rectum) was measured using a macroscopic score (1: solid granular, 2:
114 firm, 3: soft, 4: pulpy, 5: liquid, 6: watery, (Szabo et al., 2009)).

115 In the second experimental part (2), twelve animals were randomly selected out of the
116 five litters mentioned above (including six animals from experimental part 1 which were
117 sacrificed at day 28 p.i.) and were tested on day 5 prior to infection (ante infectionem, a.i.),
118 day 0 (the day of infection, samples were taken approximately 3 hours a.i.) and days 1, 2, 3, 6,
119 9, 15, 21 and 28 p.i. (33 days time series) for rectal Enterobacteriaceae populations. Rectal
120 samples were taken by sterile cotton swabs. During the observation period of experimental
121 part 2, clinical parameters of the 12 animals were monitored for (1) weight (twice weekly), (2)
122 evaluation of general condition and fecal consistency which was measured daily using a
123 macroscopic score from 1 to 6 (see above), and (3) rectal temperature (daily).

124

125 **Definitions of Enterobacteriaceae populations.** Four groups of Enterobacteriaceae were
126 distinguished for clear representation of data: (1) the total *E. coli* population, (2) the
127 *Salmonella* population, (3) total non-*Salmonella* Enterobacteriaceae (NSE) including all
128 Enterobacteriaceae other than *Salmonella*, and (4) non-*E. coli*/non-*Salmonella*

129 Enterobacteriaceae (NENSE) including all Enterobacteriaceae other than *E. coli* and
130 *Salmonella*. Although the NENSE population consisted of many different species, this
131 approach was chosen because several species occurred only very rarely and thus a separate
132 statistical analysis for each species was not useful. However, if a genus/species was isolated
133 and defined more than 5 times in an intestinal section, a separate statistical analysis was
134 additionally included for this genus/species.

135

136 **Cultivation of Enterobacteriaceae.** Enterobacteriaceae from sacrificed piglets were isolated
137 from intestinal contents and from mucosal tissues from either the distal jejunum (50 cm
138 proximal to the *Plica ileocaecalis*) or proximal to the ileo-caecal opening. The distal jejunum
139 was chosen as representative of the small intestine since in previous studies the more
140 proximal jejunum generally contained no intestinal contents and bacteria of distal jejunum are
141 thought to be similar to bacteria of the ileum but distinct from the colon (Dixit et al., 2004).
142 The proximal colon was chosen as part of the large intestine as other authors found uniformity
143 of bacterial adhesion at a number of sites of the large intestine indicating that small biopsy
144 samples are likely to be representative of larger areas of the gut epithelium (Hartley et al.,
145 1979; Swidsinski et al., 2002).

146 Piglets were euthanized by injection with sodium pentobarbital, and clamped intestinal
147 sections were removed for bacterial isolations from intestinal contents. Intestinal contents
148 were plated by serial dilutions on CHROMagar orientation agar (Merlino et al., 1996),
149 MacConkey agar for lactose fermentation (Oxoid, Hampshire, UK), and Xylose-Lysine-
150 Desoxycholat agar (XLD, Oxoid, Hampshire, UK). Bacteria from the mucosa were isolated
151 from an approximately 2 x 5 cm section of intestinal tissue of each intestinal sample washed
152 twice in phosphate-buffered saline (PBS) to remove visible fecal material. These short
153 washing steps were not expected to affect mucosa-attached bacteria as mucosa- or epithelial

154 cell-associated *E. coli* remain adherent after up to four to six changes of physiological saline
155 washes (Hartley et al., 1979; Swidsinski et al., 2002). Approximately 0.5 g of each mucosal
156 sample was scraped from connective tissue with glass slides. Mucosal samples were
157 transferred to a Dounce-homogenizer, were homogenized in 5 mL 1X PBS and serial
158 dilutions were plated on agar plates. Tubes containing rectal samples (containing
159 approximately 0.1 g rectal content on a cotton swab) from the Experimental part 2 were filled
160 with 3 ml 1X PBS and vortexed for 1min at 3000 rpm. Serial dilutions were plated on agar
161 plates as described above.

162 Intestinal contents (digesta samples) and mucosal tissues (mucosal samples) were plated
163 in parallel on CHROMagar orientation agar, MacConkey agar and XLD agar plates to obtain
164 an overview of the composition of the total Enterobacteriaceae population. The absolute
165 numbers of coliform bacteria were on average lower on MacConkey and XLD agar plates
166 compared to CHROMagar orientation agar plates (data not shown). For comparability of data
167 from several samples we chose to enumerate *E. coli* and other non-*Salmonella*
168 Enterobacteriaceae (NSE) on the basis of the least selective CHROMagar orientation agar
169 plates only.

170 The determination of populations of *E. coli* (pink coliform colonies) and for non-*E.*
171 *coli*/non-*Salmonella* Enterobacteriaceae (NENSE, defined as blue to dark violet
172 Enterobacteriaceae-like colonies in this study) was done by using CHROMagar orientation
173 agar plates. These agar plates enable the determination of both *E. coli* as well as NENSE
174 numbers (Alali et al., 2008; Merlino et al., 1996; Schierack et al., 2007). Determinations of
175 *Salmonella* (pink colonies with a black central spot) were based on XLD agar plates only.
176 Hemolytic activity of *E. coli* colonies was tested on sheep blood agar plates. If the NENSE
177 isolates of one intestinal sample comprised more than 5% of NSE, one representative NENSE
178 isolate of one intestinal sample was verified using API 20 E® (Biomerieux, France) tests for

179 further species identification. Our definition of the 5% cut-off point to define a single NENSE
180 species by the API system was based on the practicability of verification due to the common
181 occurrence of NENSE below this cut-off point (see Supplementary Figure S1). However, for
182 calculations of correlations between *E. coli*, NENSE and *Salmonella*, exact numbers for all
183 three populations were included. *Salmonella*-like colonies were additionally examined for
184 their green fluorescence on XLD agar plates.

185 Data from a mucosal sample (mucosa-associated bacteria) comprised adherent as well
186 as intracellular bacteria. Data from a digesta samples comprised bacteria of the intestinal
187 contents. Data from an intestinal section includes data from both the digesta and the mucosal
188 sample of this section. Data from a rectal sample comprised bacteria of the rectal contents.

189

190 **Statistical analysis.** Statistical analysis was performed using SPSS 12.0.2 (SPSS Inc.). In all
191 cases, non-parametric statistical tests were applied. Associations between ordinal or
192 metrically scaled parameters were tested by application of the Spearman rank correlation test
193 procedure. Differences in central tendencies between two sample groups were analyzed by
194 application of the Mann-Whitney-U test (independent samples) or the Wilcoxon-test (paired
195 samples). In general, the level of significance α was set to 0.05. In Experimental part 2, data
196 correlations were separately analyzed for each time point and were only considered relevant
197 when statistical tests were significant at least half the time. As this study was intended to
198 provide a descriptive analysis of the relationships between microbial parameters, a correction
199 for the effects of multiple testing was not applied. Nevertheless, while results with p-values
200 below 0.01 are indicated as additional information, in general, the results of this study should
201 be considered as descriptive in nature.

202

202 **Results**

203 **Enterobacteriaceae-general comments.** NSE were detected in all colonic and rectal
204 samples. In contrast, in several jejunal samples (digesta (n=1/40) or mucosa (n=5/40)) NSE
205 were not detectable. If NSE were isolated from an intestinal section, *E. coli* were always
206 present. However, there were two jejunal mucosa and one jejunal digesta samples with only
207 *Klebsiella pneumoniae* or *Klebsiella* spp. or *Enterobacter cloacae*, but with *E. coli* in the
208 other compartment of a given section.

209 The following NENSE (non-*E. coli*/ non-*Salmonella* Enterobacteriaceae) species and
210 genera were isolated from jejunal and/or colonic and/or rectal samples: *Citrobacter freundii*
211 (up to 90.7% of the total NSE population of a single sample), *Enterobacter cloacae* (100%),
212 *Enterobacter sakazakii* (93%), *Klebsiella ornithinolytica* (36%), *Klebsiella pneumoniae*
213 (100%), *Klebsiella oxytoca* (10.9%), *Klebsiella* spp. (100%), *Pantoea* spp. (68.1%), *Rahnella*
214 *aquatilis* (15.4%), *Serratia marcescens* (84.9%) and *Serratia* spp. (91.3%).

215 Hemolytic *E. coli* were sporadically present in all piglet groups and occurred in colonic
216 and/or jejunal and/or rectal samples. However, the occurrence of hemolytic *E. coli* had no
217 obvious effects on the occurrence of other *E. coli*, NENSE or *Salmonella*. In addition, the
218 isolation frequency of hemolytic *E. coli* did not change after *Salmonella* infection.

219

220 **Experimental part 1. Analysis of Enterobacteriaceae in digesta and mucosal samples of**
221 **killed pigs.** The absolute counts of *E. coli* numbers in the jejunum and colon, NENSE
222 numbers in the jejunum digesta and the absolute *Salmonella* counts in the mucosa of the
223 jejunum and colon decreased significantly with the age of the animals (Table 1).

224 There were in general higher numbers of *E. coli* in the colon compared to the jejunum
225 ($p < 0.01$), but no differences for NENSE and *Salmonella*. In addition, there were higher
226 numbers of *E. coli* and NENSE in the digesta compared to mucosa (jejunum as well as colon,

227 all tests with p-values < 0.01) and higher numbers of *Salmonella* in the digesta compared to
228 mucosa (p<0.01). Additionally, high numbers of mucosa-adherent bacteria of one population
229 correlated with high numbers of bacteria of this population in the digesta (Fig. 1).

230 In the jejunum the numbers of isolated *E. coli*, NENSE and *Salmonella* were highly
231 positively intercorrelated, “*i.e.*” if a jejunum sample showed a high number of NENSE, there
232 was a higher probability of observing high numbers of *E. coli* or *Salmonella* as well. In the
233 colon, samples with this strong intercorrelation pattern were less frequently observed although
234 some correlations were statistically significant (*E. coli* vs. *Salmonella* in colon digesta:
235 p<0.05; NENSE vs. *Salmonella* in colon digesta: p<0.05) (Fig. 2).

236 The frequency of isolated single NENSE species is shown in Table 2. No single NENSE
237 genus/species was isolated more than 5 times in the colon. *Klebsiella* was the only genus
238 which was sampled and defined more than 5 times in the jejunum, a separate statistical
239 analysis was performed for this genus. However, the occurrence of *Klebsiella* did not
240 correlate with *E. coli* and *Salmonella* populations of the jejunum.

241 The occurrence of *Salmonella* in jejunum as well as in colon samples varied
242 considerably between individual pigs even at 3h p.i. For example, in a single pig (animal No.8
243 in Fig. 3) we detected no *Salmonella* in the jejunum or colon (digesta or mucosa) at this time
244 point. In contrast, only two other animals had *Salmonella* in the jejunum and colon (digesta
245 and mucosa, animals No. 3 and 9 in Fig. 3). The highest number of *Salmonella*-positive
246 samples was detected at 24 h p.i. (Table 3).

247

248 **Experimental part 1. Associations between numbers of Enterobacteriaceae in digesta**
249 **and mucosal samples of killed pigs and disease-related parameters.** There were no
250 significant dependencies detectable between the numbers of *E. coli* or NENSE in digesta or
251 mucosal samples and the body temperatures of animals. However, lower numbers of

252 *Salmonella* in colonic digesta samples were associated with elevated body temperatures of the
253 animals ($p < 0.01$). This correlation remained significant even after correcting for the influence
254 of the animal age by application of the partial correlation analysis algorithm on *Salmonella*
255 non-negative samples.

256 Fecal consistencies were significantly positively associated with *E. coli* counts from the
257 colon digesta and mucosal samples and colon digesta consistencies were significantly
258 associated with *E. coli* counts from the colon digesta samples, “i.e.” the more *E. coli* were
259 detected the more solid the digesta and feces were (all $p < 0.05$). In contrast, where NENSE
260 were detectable in jejunal digesta samples, high NENSE counts were negatively associated
261 with digesta consistencies, “i.e.” the more NENSE were detected the more liquid the digesta
262 was ($p = 0.074$ for the samples from digesta, $p < 0.05$ for samples from the mucosa). The
263 number of *Salmonella* was not significantly associated with digesta consistencies in the colon
264 or jejunum, but *Salmonella* from colon digesta and jejunum mucosa showed a strong positive
265 correlation with solid feces, as was observed for *E. coli* counts ($p < 0.01$). As *Klebsiella* was
266 the only genus which was sampled and defined more than 5 times in the jejunum, a separate
267 statistical analysis was performed for this genus. However, the occurrence of *Klebsiella* did
268 not correlate with digesta consistencies or body temperatures of animals.

269

270 **Experimental part 2. Analysis of Enterobacteriaceae in rectal samples of live piglets**
271 **during a 33 days time series.** The absolute *E. coli* counts of rectal samples decreased
272 significantly with the age of the animals ($p < 0.01$), whereas for the absolute NENSE counts
273 such time-dependency could not be verified due to the high percentage of NENSE-negative
274 samples. When analyzing the intercorrelations between *E. coli*, *Salmonella* and NENSE
275 numbers separately at each time point of this time series, the occurrence of high numbers of
276 isolates of a single Enterobacteriaceae-population was not correlated to high numbers of

277 another population, and single NENSE species could not be correlated with other
278 Enterobacteriaceae populations due to low abundances of these species.

279 The occurrence of *Salmonella* in rectal samples varied considerably between individual
280 pigs. An apparent increase of *Salmonella* shedding was observed between 3 h and 24 h p.i.
281 followed by a decrease of *Salmonella* shedding after 24 h until day 28 p.i. ($p < 0.01$). The level
282 of *Salmonella* shedding in feces during the three days following the infection (24 h to 72 h
283 p.i.) varied between 3 to 8 log units (log(cfu/g)).

284

285 **Experimental part 2. Associations between numbers of Enterobacteriaceae in rectal**
286 **samples and disease-related parameters during a 33 day time series.** During the
287 observation period of 28 days post infection, the weight of animals increased continuously,
288 corresponding to the normal development of body weight in healthy, weaning pigs. Elevated
289 body temperature over 40.0°C was observed in 33.3% (4/12) of the animals during the four
290 week observation period. The fecal consistency of all samples before infection was soft to
291 solid (scores 2-3). Diarrhea was observed in 16.7% (2/12) of animals within the first three
292 days p.i. and in 41.6% (5/12) of animals within the four weeks observation period (score
293 5=liquid feces). However, the bacterial counts taken during this time showed no significant
294 dependencies between the numbers of *E. coli* or NENSE or *Salmonella* in rectal samples and
295 either the fecal consistencies or body temperatures of animals. Individual NENSE species
296 could not be correlated with disease-related parameters due to low abundances of these
297 species.

298

298 **Discussion**

299 With the exception of *E. coli*, the composition of the autochthone porcine intestinal
300 Enterobacteriaceae microflora has been only partially studied (Schierack et al., 2007), and
301 changes during a *Salmonella* infection remained unknown. In this study, we determined the
302 dynamics of the autochthone *Enterobacteriaceae* microflora and investigated possible
303 relationships between the occurrence of intestinal *Salmonella* and the occurrence of other
304 Enterobacteriaceae during an experimental *Salmonella* infection. The animal infections were
305 performed with *Salmonella* Typhimurium phagetype DT104, one of the most frequently
306 isolated *Salmonella* serovar in pork, and a common *Salmonella* serovar isolated from a wide
307 range of other animals and humans and of particular concern because of its acquisition of
308 multiple antibiotic resistances (Baggesen and Aarestrup, 1998; Poppe et al., 2002; Threlfall et
309 al., 2000). Our goal was to monitor infection-dependent changes and possible protective
310 functions of ratios of members of the autochthone Enterobacteriaceae microflora against a
311 *Salmonella* infection. We also performed correlation analyses for the occurrences of *E. coli*,
312 non-*E. coli*/non-*Salmonella* Enterobacteriaceae (NENSE) and *Salmonella* with alterations in
313 clinical parameters which were also monitored during an experimental subclinical to mild
314 Salmonellosis.

315 In our study, a specific ratio between the *E. coli* and the total NENSE population as well
316 as the occurrence of single NENSE genera/species were not correlated with low numbers of
317 *Salmonella* and thus appeared to have no protective function against the *Salmonella* infection.
318 In contrast, high *Salmonella* counts were accompanied by high numbers of non-*Salmonella*
319 Enterobacteriaceae (NSE) and high numbers of mucosa-associated *Salmonella* were also
320 correlated with high numbers of mucosa-attached *E. coli*. Such a rather proportional than
321 competing effect was also seen comparing the *E. coli* and the total NENSE population as high
322 numbers of NENSE were also markedly correlated with high numbers of *E. coli*. These results

323 suggest either (1) each Enterobacteriaceae population supported the occurrence of other
324 Enterobacteriaceae or (2), the intestinal milieu affected the intrainestinal growth of all
325 Enterobacteriaceae populations to a similar extent.

326 During the course of the infection study, the absolute numbers of *E. coli*, NENSE and
327 *Salmonella* continuously decreased in rectal samples. This decrease was associated with a
328 decrease of these Enterobacteriaceae populations in both the jejunum as well as in the colon.
329 Comparison of the data from this study with a recent study of non-infected piglets of the same
330 pig population (Scharek et al., 2005), indicated that the decrease of coliform bacteria after
331 *Salmonella* infection was no more prominent than the decrease of coliform bacteria in non-
332 infected animals. Such an age-dependent decrease of coliform bacteria seems to be common
333 in the fast developing intestines of piglets (Katouli et al., 1999). In summary, the *Salmonella*
334 infection did not appear to result in a marked reduction or increase of NSE. However, a
335 decrease in the rectal populations might reflect a decrease of the jejunal or colonic
336 Enterobacteriaceae populations.

337 We observed a correlation between numbers of bacteria from an Enterobacteriaceae
338 population and the consistency of the digesta samples. The colon digesta and feces
339 consistencies were associated with *E. coli* counts from the colon, “i.e.” the more *E. coli* were
340 detectable the more solid the digesta and feces were. A similar correlation was found for
341 *Salmonella* in the jejunum and colon and the consistency of animal feces. In contrast, the
342 more NENSE detectable in the jejunum, the more liquid the digesta was. These correlations
343 might be explained by two possibilities: (1) The changes in fecal/digesta consistency may
344 have been due to changes of the intestinal milieu which in turn reflected changes of the
345 reabsorption/excretion capacity of the mucosa. The Enterobacteriaceae populations might be
346 expected to adapt to such intestinal alterations with increased/decreased numbers of an
347 Enterobacteriaceae population reflecting the consequences of the changes of the intestinal

348 milieu. (2) Alternatively, certain Enterobacteriaceae populations may increase/decrease in the
349 intestine in response to intestinal disorders. These increased/decreased numbers of bacterial
350 population(s) may affect the intestinal mucosa and cause the changes in fecal/digesta
351 consistency. Whether changes in the autochthone Enterobacteriaceae populations are the
352 cause or result from changes in intestinal physiology and/or disorders remains speculative.

353 Changes in the intestinal milieu including weaning, changes in feeding regime and
354 intestinal diseases (e.g. viral diseases) are thought to support the emergence or dominance of
355 pathogenic hemolytic *E. coli* (Franklin et al., 2002; Hampson et al., 1985; Lecce et al., 1982;
356 Pluske et al., 2007). Although *Salmonella* infection led to mild diarrhea in several animals in
357 our study and hemolytic *E. coli* were present in all piglet groups, an increase or a dominance
358 of hemolytic *E. coli* were not detectable. We conclude that hemolytic *E. coli* already present
359 in the intestinal microflora did not exploit or reinforce the intestinal disturbances caused by
360 the *Salmonella* infections (Szabo et al., 2009).

361 Identical experimental *Salmonella* infection regimes resulted in often very different
362 infection success as determined by intestinal counts in different animals at different time
363 points. Thus a successful *Salmonella* infection appears to be strongly dependent on the
364 individual status of a given animal. During intestinal *Salmonella* infection experiments in
365 swine, the numbers of *Salmonella* in the intestine therefore have to be constantly monitored
366 as a reference point to evaluate individual effects on other microbial, biochemical or
367 immunological parameters.

368 In this study we have made a number of additional general observations which may or
369 may not be independent of a *Salmonella* infection. Here we report for the first time the
370 isolation of several Enterobacteriaceae species from the pig (*Enterobacter sakazakii*,
371 *Klebsiella ornithinolytica*, *Klebsiella oxytoca*, *Rahnella aquatilis* and *Serratia marcescens*).
372 This confirmed our previous assumptions that the porcine intestine is a natural habitat for a

373 broad variety of different Enterobacteriaceae species (Schierack et al., 2007). However, also
374 in this study, *E. coli* was the clearly most dominant Enterobacteriaceae species in the jejunum,
375 the colon as well as in the rectum (Schierack et al., 2007).

376 As shown in a previous study, there were higher numbers of Enterobacteriaceae in the
377 colon compared to the jejunum and higher numbers of Enterobacteriaceae in the digesta
378 compared to the mucosa samples (Schierack et al., 2007). However, as also noted in this
379 previous study, there were also determinations showing no Enterobacteriaceae in the jejunum.

380 For the determination of Enterobacteriaceae numbers we compared plating results on
381 different agar plates. Absolute NSE numbers were in general lower on MacConkey, XLD
382 agar and also on the initially used Gassner agar plates compared to CHROMagar orientation
383 agar plates. However, differences in absolute numbers differed from sample to sample. We
384 therefore chose to only use absolute numbers of *E. coli* and non-*E. coli*/non-*Salmonella*
385 Enterobacteriaceae as determined from CHROMagar orientation agar plates (the less
386 restrictive agar plate). CHROMagar orientation agar plates identify by 99.3% *E. coli* isolates
387 due to their pink/red colony colors and are generally accepted to be the only plate type
388 sufficiently capable of differentiating *E. coli* from other Enterobacteriaceae (Alali et al., 2008;
389 Merlino et al., 1996; Schierack et al., 2008; Schierack et al., 2007). CHROMagar orientation
390 agar plates were also the basis for our definition of NENSE. Due to practical reasons here we
391 focused on blue/violet coliform colonies. A broad spectrum of Enterobacteriaceae has been
392 shown to form blue/violet coliform colonies on CHROMagar orientation agar plates with
393 different shades of color: several species of the genera *Buttiauxella*, *Citrobacter*,
394 *Enterobacter*, *Hafnia*, *Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, *Yersinia* (Merlino
395 et al., 1996; Schierack et al., 2007 and unpublished results). However, we are aware of that
396 other members of the Enterobacteriaceae population might be excluded from our analysis as
397 there might have been Enterobacteriaceae genera/species which do not form pink/red or

398 blue/violet colonies on CHROMagar orientation agar plates. As we also plated intestinal
399 samples to MacConkey and XLD plates (and initially Gassner agar plates), we had the
400 possibility to observe colony differences between NENSE isolates (color, size, surface etc.).
401 However, NENSE isolates of a given sample were always very similar on agar plates. We
402 conclude that the dominant fraction of the NENSE population of one intestinal sample
403 consists largely of one species, as suggested in a prior study (Schierack et al., 2007).

404 Based on the results of this study, we find no correlations or changes in the ratios of
405 single or groups of Enterobacteriaceae populations which would provide predictive or
406 diagnostic markers based on the composition of the Enterobacteriaceae population for
407 asymptomatic or subclinical *Salmonella* infections. Rather, we observed a similar trend for
408 the endogenous populations concerning absolute numbers of *E. coli* and NENSE following
409 experimental introduction of *Salmonella* into the intestinal microflora. In addition,
410 endogenous hemolytic *E. coli* did not appear to play a role during an experimental *Salmonella*
411 infection. Clearly the interplay between intestinal microflora populations is complex, but it
412 would appear that these populations are also rather stable, since introduction of a
413 diarrheagenic intestinal pathogen such as *Salmonella* did not result in perturbations resulting
414 in significant shifts in populations or even single genera.

415

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420

Accepted Manuscript

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499 **Figure captions**

500 **Fig. 1.** Correlations between numbers of mucosa-adherent bacteria and bacteria from the
501 digesta in the jejunum and colon of killed piglets (n=40, Experimental part 1.). High numbers
502 of mucosa-adherent bacteria correlated with high numbers of bacteria from the digesta. A:
503 jejunum, B: colon.

504

505 **Fig. 2.** Correlations between numbers of *E. coli* and NENSE (A), *E. coli* and *Salmonella* (B)
506 and NENSE and *Salmonella* (C) in the jejunum and colon of killed piglets (n=40,
507 Experimental part 1.). High numbers of *E. coli* correlated with high numbers of NENSE and
508 *Salmonella*, high numbers of NENSE correlated with high numbers of *Salmonella* in the
509 jejunum.

510

511 **Fig. 3.** Individuality of *Salmonella* numbers in the jejunum and colon of killed piglets (n=10)
512 3 h after *Salmonella* infection (Experimental part 1.). A: jejunum digesta, B: jejunum mucosa,
513 C: colon digesta and D: colon mucosa.

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526 Fig. 1.

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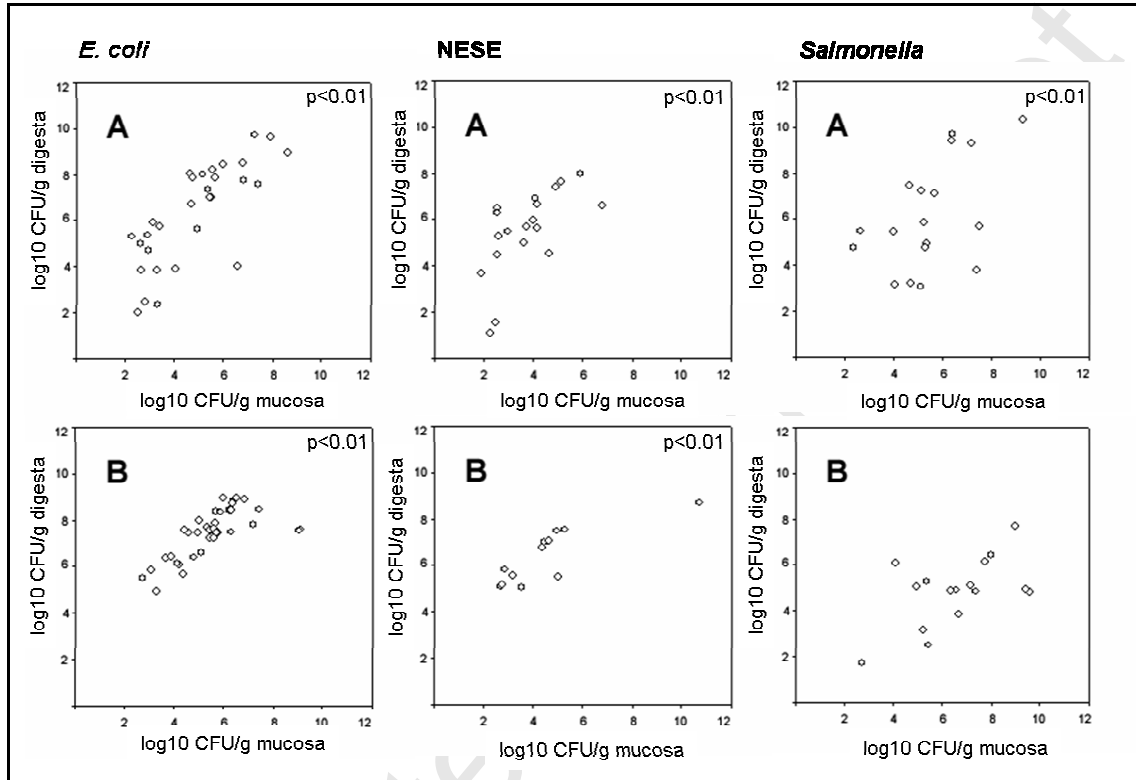
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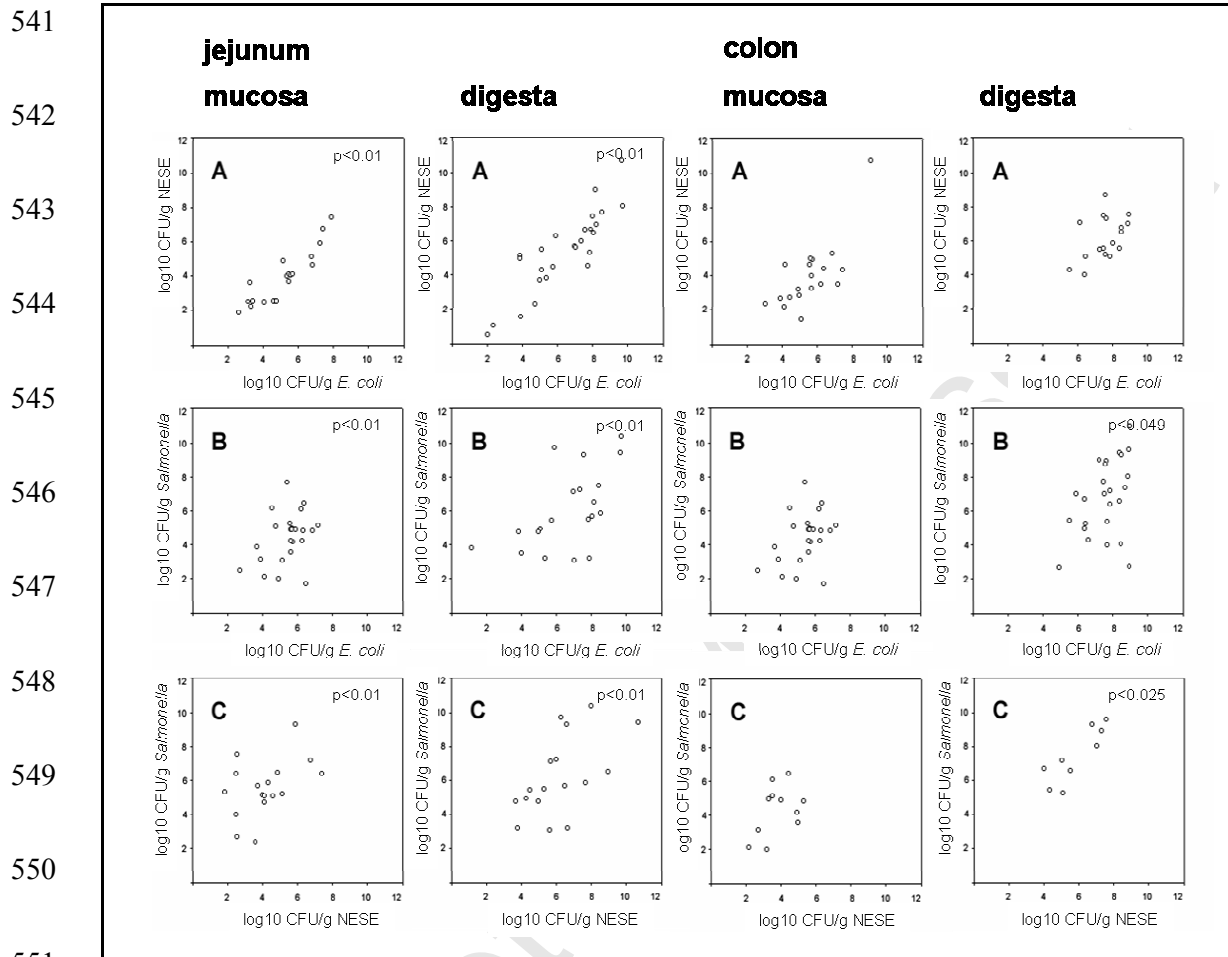
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540 Fig. 2.



553 Fig. 3.

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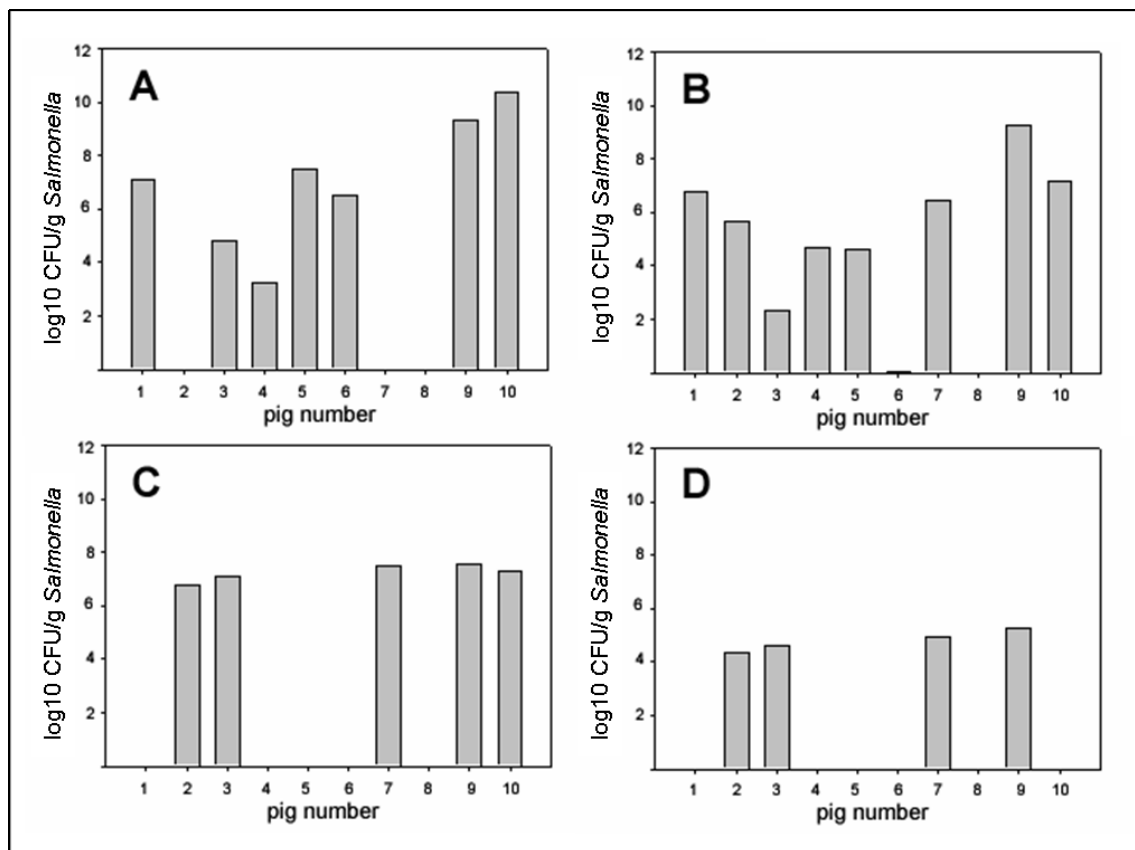
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579 **Tables**

580 **Table 1. Significance (p) of the (negative) correlation between age and absolute *E. coli*,**
 581 **NENSE or *Salmonella* numbers after *Salmonella* infection in the jejunum and colon of**
 582 **killed piglets (n=40, Experimental part 1.) and in the rectum of live piglets during a 33**
 583 **days time series (n=12, Experimental part 2.).**

584

585

| | significance (p) | | | | |
|-------------------|------------------|---------|---------|--------|----------|
| | jejunum | jejunum | colon | colon | rectum |
| | digesta | mucosa | digesta | mucosa | |
| | (1%) | (1) | (1) | (1) | (2) |
| <i>E. coli</i> | <0.05 | <0.01 | <0.01 | <0.01 | <0.01 |
| NENSE | <0.01* | n.s.* | n.s.* | n.s.* | =0.056*§ |
| <i>Salmonella</i> | n.s.# | <0.05# | <0.01# | <0.01# | <0.01# |

586

587 % Experimental part

588 * only NENSE-positive samples are included

589 # only *Salmonella*-positive samples are included

590 n.s. not significant

591 § tendency

592

592 **Table 2. The occurrence of non-*E. coli*/non-*Salmonella* Enterobacteriaceae (NENSE) in**
 593 **the jejunum and colon of killed piglets (n=40, Experimental part 1.) and in the rectum of**
 594 **live piglets during a 33 days time series (n=12, Experimental part 2.).**

| genus/species* | no. of samples positive for this genus/species | | | | | | |
|---------------------------|--|---------|---------|--------|---------|--------------|--------------|
| | jejunum | jejunum | colon | colon | rectum§ | total no. of | total no. of |
| | digesta | mucosa | digesta | mucosa | | samples | animals |
| | (1#) | (1) | (1) | (1) | (2) | (1+2) | (1+2) |
| <i>C. freundii</i> | 2 | 2 | 2 | 0 | 3 (1)§ | 9 | 5 |
| <i>E. cloacae</i> | 1 | 1 | 1 | 1 | 0 | 4 | 2 |
| <i>E. sakazakii</i> | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| <i>K. ornithinolytica</i> | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| <i>K. pneumoniae</i> | 7 | 2 | 0 | 4 | 2 (2) | 15 | 12 |
| <i>K. oxytoca</i> | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| <i>Klebsiella</i> spp. | 2 | 0 | 0 | 0 | 0 | 2 | 2 |
| <i>Pantoea</i> spp. | 0 | 1 | 1 | 0 | 0 | 2 | 2 |
| <i>R. aquatilis</i> | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| <i>S. marcescens</i> | 0 | 0 | 0 | 0 | 2 (2) | 2 | 2 |
| <i>Serratia</i> spp. | 1 | 1 | 1 | 1 | 0 | 4 | 1 |

595

596 * *C.*: *Citrobacter*; *E.*: *Enterobacter*; *K.*: *Klebsiella*; *R.*: *Rahnella*; *S.*: *Serratia*

597 # Experimental Part

598 § since each animal was tested during a time-period, the number of animals positive for a
 599 genus/species is included in parentheses

600

600 **Table 3. Prevalence of *Salmonella* in the jejunum and colon of killed piglets (n=40,**
 601 **Experimental part 1.) and in the rectum of live piglets during a 33 days time series**
 602 **(n=12, Experimental part 2.).**

| time after infection | prevalence (%) | | | | | |
|-------------------------|---------------------|--------------------|-------------------|------------------|--|---------|
| | jejunum digesta* | jejunum mucosa* | colon digesta* | colon mucosa* | none salmonella in jejunum and colon* | rectum* |
| | (1#) | (1) | (1) | (1) | (1) | (2) |
| 3 h | 50 | 50 | 50 | 40 | 20 | n.d.§ |
| 24 h | 80 | 90 | 80 | 80 | 0 | 100 |
| 72 h | 50 | 80 | 70 | 80 | 0 | 92 |
| 28 d | 20 | 30 | 50 | 40 | 10 | 42 |

603

604 * after direct plating of samples on agar plates (no *Salmonella* enrichment)

605 # Experimental Part

606 § not determined

607 .

608

609